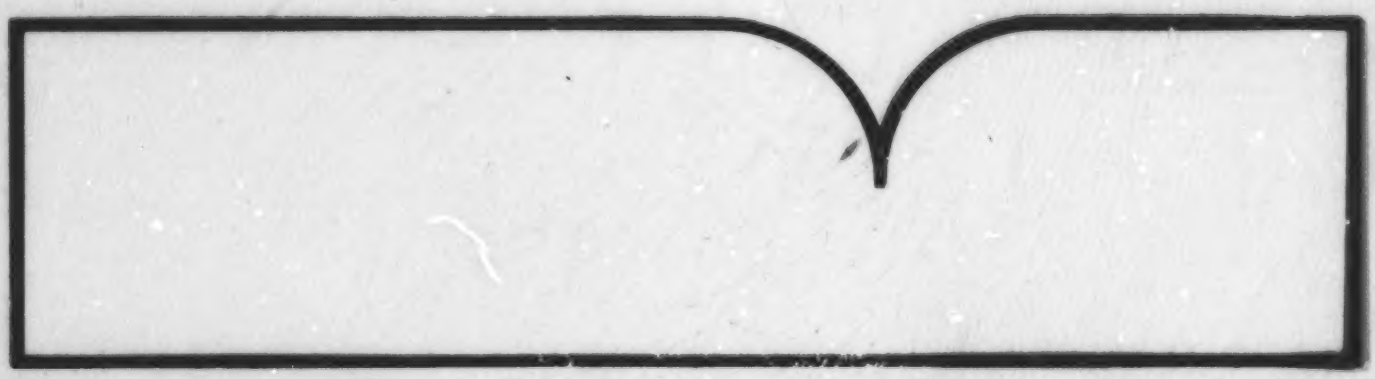
PB84-238674

Effect of Benomyl on the Reproductive Development of Male Rats

(U.S.) Health Effects Research Lab.
Research Triangle Park, NC

1984



U.S. Department of Commerce
National Technical Information Service
NTIS

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO. EPA-600/J-84-077	2.
3. R	PB84-238674
4. TITLE AND SUBTITLE: Effect of Benomyl on the Reproductive Development of Male Rats (Journal Version)	5. REPORT DATE: 1984
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S): Susan D. Carter, Joy F. Hein, Georgia L. Rehnberg, and John W. Laskey	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Developmental Biology Division, Health Effects Research Laboratory, US Environmental Protection Agency, Research Triangle Park, NC 27711	10. PROGRAM ELEMENT NO. A9RA1B
	11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS: Health Effects Research Laboratory, Office of Research and Development, US Environmental Protection Agency, Research Triangle Park, NC 27711	13. TYPE OF REPORT AND PERIOD COVERED
	14. SPONSORING AGENCY CODE EPA-600/11

15. SUPPLEMENTARY NOTES

Published In: Journal of Toxicology and Environmental Health 13: 53-68, 1984

16. ABSTRACT

Benomal, a systemic fungicide, was administered to male Sprague-Dawley rats during the prepubertal, pubertal, or postpubertal stage of reproductive development. Animals received 5 or 10 daily treatments of 0, 125, 200, 250, 500, or 1000 mg benomyl/kg·d by gavage. Observations were made at selected intervals after exposure and included hemotological parameters, body weight, tissue weights, total epididymal sperm counts, vas deferens sperm concentrations, serum follicle-stimulating hormone (sFSH) levels, and testicular histology. Data presented here suggest that there is an age-related difference in sensitivity to benomal. Animals that received benomal treatments during prepuberty showed no significant effects in tissue weights, total epididymalsperm counts, vas deferens sperm concentration, or sFSH. Animals that received at least 250 mg/kg·d during puberty or postpuberty showed one or more of the following effects: decreased testicular or epididymal weights, decreased epididymal sperm count, decreased vas deferens sperm concentrations, and/or testicular lesions. Histological examination of testicular tissue indicated a higher incidence of diffuse hypospermato-cytogenesis in pubertal (20% of the treated animals) and postpubertal (40% of the treated animals) animals that were exposed to benomyl. These values were compared with those of the treated prepubertal animals, which had a 10% incidence of diffuse hypospermatogenesis, and with all of the control animals, which had no occurences of testicular lesions.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

18. DISTRIBUTION STATEMENT: Release to Public	19. SECURITY CLASS (This Report): Unclassified	21. NO. OF PAGES: 16
	20. SECURITY CLASS (This page): Unclassified	22. PRICE

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

EPA-600/J-84-077
JOURNAL ARTICLE

EFFECT OF BENOMYL ON THE REPRODUCTIVE DEVELOPMENT OF MALE RATS

Susan D. Carter, Joy F. Hein, Georgia L. Rehnberg, John W. Laskey

Environmental Protection Agency, Health Effects Research Laboratory, Developmental Biology Division, Research Triangle Park, North Carolina

Benomyl, a systemic fungicide, was administered to male Sprague-Dawley rats during the prepuberal, pubertal, or postpubertal stage of reproductive development. Animals received 5 or 10 daily treatments of 0, 125, 200, 250, 500, or 1000 mg benomyl/kg·d by gavage. Observations were made at selected intervals after exposure and included hematological parameters, body weight, tissue weights, total epididymal sperm counts, vas deferens sperm concentration, serum follicle-stimulating hormone (sFSH) levels, and testicular histology. Data presented here suggest that there is an age-related difference in sensitivity to benomyl. Animals that received benomyl treatments during prepuberty showed no significant treatment effects in tissue weights, total epididymal sperm counts, vas deferens sperm concentration, or sFSH. Animals that received at least 250 mg/kg·d during puberty or postpuberty showed one or more of the following effects: decreased testicular or epididymal weights, decreased epididymal sperm count, decreased vas deferens sperm concentrations, and/or testicular lesions. Histological examination of testicular tissue indicated a higher incidence of diffuse hypospermatocytogenesis in pubertal (20% of the treated animals) and postpubertal (40% of the treated animals) animals that were exposed to benomyl. These values were compared with those of the treated prepubertal animals, which had a 10% incidence of diffuse hypospermatocytogenesis, and with all of the control animals, which had no occurrences of this testicular lesion.

INTRODUCTION

The extensive use of benomyl, methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate (Dupont Benlate benomyl fungicide), as a systemic fungicide to control a wide variety of plant pathogens has stimulated concern as to its potential risks to humans. Benomyl inhibits microtubule formation in fungi and to a much lesser extent in plants and mammals by binding to tubulin and thereby preventing tubulin polymerization (Davidse and Flach,

This paper has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

The authors gratefully acknowledge the National Institute of Arthritis, Metabolism and Digestive Diseases for providing materials for the sFSH radioimmunoassay, and Dr. Thomas M. Scotti for his consultation and assistance with the histology and photomicrographs.

Requests for reprints should be sent to Susan D. Carter (M-72), Environmental Protection Agency, Health Effects Research Laboratory, Research Triangle Park, NC 27711.

1977; Ireland et al., 1979). Major consequences of microtubule interferences are inhibition of cellular processes, such as development and maintenance of cell shape, intracellular transport, and chromosomal movements during cell divisions (Olmstead and Borisy, 1973). This inhibition results in nonfunctioning cells and death in most fungi. The binding affinity of benomyl with mammalian tubulin is much lower than the affinity that occurs with fungal tubulin (Ireland et al., 1979). However, when large concentrations of benomyl are present, the benomyl–mammalian tubulin complexes form and further polymerization is inhibited (Friedman and Platzer, 1978). In cases where increased synthesis and utilization of microtubules are required, such as during rapid cellular divisions occurring with growth, spermatogenesis, and hematopoiesis, benomyl exposure may be potentially toxic.

The effects of benomyl on reproduction resulting from chronic dietary or acute oral exposures have been investigated in laboratory animals. Sherman et al. (1975) conducted a three-generation reproduction study in which male and female rats were fed diets containing 0, 0.01, 0.05, or 0.25% benomyl. No treatment differences were noted in reproductive performance (i.e., fertility, gestation, viability, and lactation indices) in any generation. However, other studies indicated that benomyl or its metabolites reach the testes and are capable of causing degeneration of testicular tissue (Torchinskiy et al., 1976; Carter and Laskey, 1982). Decreased testicular weight and the absence of spermatozoa within the seminiferous tubules were seen in rats after chronic (1000 mg benomyl/kg·d) exposure (Torchinskiy et al., 1976). Kavlock et al. (1982) reported lower testicular weights in 100-d-old Wistar rats that had received 31.2 mg/kg·d by gavage during the perinatal period. Carter and Laskey (1982) have reported a 32–48% reduction in vas deferens sperm concentration along with damage to the testicular germinal epithelium in adult Sprague-Dawley rats after 10 daily exposures to benomyl (200 or 400 mg/kg·d).

This paper will present data from two experiments that were conducted to continue the investigation of the effects of exposure to benomyl during selected stages of male reproductive development. Here, puberty shall be defined as the age when there is full function of the reproductive organs and viable offspring may be produced. In the Sprague-Dawley rat, puberty occurs at 50–60 d of age (Fox and Laird, 1970; Hamner, 1970). Experiment 1 was designed to evaluate the reproductive development of male rats after receiving 10 daily exposures of benomyl just prior to the onset of puberty. Observations were made at 5 different time intervals (3, 17, 31, 45, and 59 d) following exposure in order to evaluate the effects of benomyl on selected stages of spermatogenesis. Given adequate interactions between hypothalamic releasing factors, pituitary gonadotrophins, and testicular androgens, spermatogenesis continues uninterrupted after its initiation by the cyclic renewal of stem spermatogonia, which give rise to new generations of spermatozoa (Gomes, 1975). Each successive cycle of spermatogenesis, beginning as stem cells and reaching mature spermatozoa, requires

approximately 45 d (Gomes, 1970). After the initial cycle has been completed, developing cells may be found in each phase of spermatogenesis at any given time. Therefore, selection of a specific postexposure observation time may allow cells in a particular phase of spermatogenesis to continue their developmental processes and travel through the seminiferous tubules to epididymides and vas deferens (Clermont and Perey, 1957; Gomes, 1970), where samples of luminal fluid may be collected. Samples obtained 3, 17, or 31 d postexposure contained spermatozoa that had been either stored in the epididymis (3 d) or was undergoing the final stages of maturation (i.e., spermiogenesis) (17 and 31 d) during exposure. Observations made at 45 and 59 d postexposure included spermatozoa that had undergone meiosis (45 d) or spermatocytogenesis (59 d) during exposure. Data from this experiment include tissue weights, measurements of total epididymal sperm and vas deferens sperm concentration, and testicular histology. Experiment 2 measured dose responses to benomyl at three different stages of reproductive development. Observations were made 31 d after the animals received 5 daily doses of benomyl during the prepubertal stage of sexual development (33–37 d of age), or just after the onset of puberty (54–58 d of age), or as an adult (postpuberty; 75–79 d of age). Data presented from this experiment include the same observations as in experiment 1. In addition, measurements were made of serum follicle stimulating hormone (sFSH) concentration and selected hematological parameters.

METHODS

Technical-grade benomyl (Fig. 1) was supplied by E. I. Dupont de Nemours and Co., Inc. (Wilmington, Del.).

Animals

For each experiment, 21-d-old Sprague-Dawley male rats were obtained from Charles River Breeding Laboratory (Kingston, N.Y.). Throughout the study all animals were maintained in a Class 100 bioclean room with controlled lighting (6 a.m. to 6 p.m.) and constant temperature (20–24°C), and were allowed food and tap water *ad libitum*.

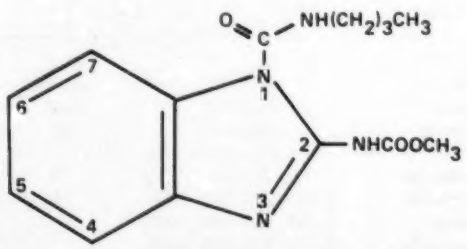


FIGURE 1. Structure of benomyl.

Experiment 1—Reproductive Development after Prepubertal Exposure

At 26 d of age the animals were randomly placed into treatment groups that received 10 daily doses (by gavage) of either 0 or 200 mg benomyl/kg·d (Table 1). Animals housed together (2 per cage) received the same treatment. To insure that degradation of the dosing suspension did not occur, suspensions of technical grade benomyl (E. I. DuPont de Nemours & Co., Inc., Wilmington, Del.) in corn oil were prepared daily. Treatment began when the animals were 33 d of age. Following the protocol used by Carter and Laskey (1982), treatments were administered by gavage 5 times a week for a period of 2 wk. Each animal was weighed prior to each treatment and dosage was adjusted for body weight.

At specific time intervals following the last treatment (3, 17, 31, 45, and 59 d), 8 animals per treatment group were weighed and then killed using CO_2 gas. Liver, kidneys, and seminal vesicles were removed and weighed. Each side of the reproductive tract, which included the testis, epididymis, and vas deferens, was removed from the abdominal cavity as one unit. To prevent fluid loss, the proximal end of the vas deferens was clamped with a hemostat prior to being cut. The testis, blood vessels, and excess fat were carefully trimmed away from the vas deferens and epididymis. Collection of luminal fluid from the vas deferens was accomplished by clamping the distal portion of the vas deferens with a hemostat, making a cut, and then gently extruding the luminal fluid onto a small piece of polystyrene weigh boat that was inserted into a 20-ml glass vial. The contents were immediately weighed and then vortexed in 2-ml of 10% phosphate-buffered formalin for 1 min. Dilutions of this mixture were used for sperm counting. The dissection of the epididymis into the caput and cauda sections was achieved by again clamping off each section prior to being cut. When using this procedure no fluid loss occurred. Sections of the epididymis were weighed, placed into 10% phosphate-buffered formalin, minced, and vortexed. Dilutions of this mixture were used for sperm counting. All sperm counts were determined using a ZBI Coulter Counter equipped with a C-1000 channelyzer (Coulter Electronics, Inc.,

TABLE 1. Experimental Design

Experiment	Reproductive development during exposure	Benomyl (mg/kg·d)	Exposure time (d)	Postexposure observation time (d)
1	Prepubertal	0, 200	10	3, 17, 31, 45, 59
2	Prepubertal	0, 125, 250, 500, 1000	5	31
	Pubertal	0, 125, 250, 500, 1000		
	Postpubertal	0, 125, 250, 500, 1000		

Hialeah, Fla.). The instrument settings, counting medium, and the dilutions used in this laboratory were obtained after modifying the methods described by Gordon et al. (1965). Estimates of the number of sperm were also calculated from repeated hemocytometer counts for 15% of the control animals and for 35% of the treated animals. These data, along with previous data recorded in this laboratory (Laws et al., 1983), indicated that the methods used produced reproducible sperm count values and that the values for the repeated hemocytometer counts were consistently within 15–18% of those values obtained using the electronic cell counter.

Testes from animals killed at selected posttreatment times (3, 17, 31, and 59 d) were fixed in Helly's fluid (Humason, 1962) for 6–7 h, washed in running water for 20 h, and stored in 70% ethanol. Testicular samples were forwarded to Experimental Pathology Laboratories, Inc. (Raleigh, N.C.), where cross sections were prepared for histological examinations using a periodic acid Schiff (PAS) stain. Criteria used to evaluate the testicular sections were as follows: (1) presence of focal hypospermatocytogenesis (i.e., occasional seminiferous tubules that were lined by Sertoli cells only or by Sertoli cells and occasional spermatogenic cells); (2) presence of diffuse hypospermatocytogenesis (i.e., significant testicular damage with more than half of seminiferous tubules per section affected); (3) presence of spermatidic giant cells (i.e., enlarged, multinucleated spermatids); (4) presence of spermatic granuloma (i.e., small lesions that consisted of localized accumulation of histiocytes, lymphocytes, and plasma cells, which are believed to represent an inflammatory cellular reaction to sperm which have escaped from disrupted tubules). Any animal with a positive classification for diffuse hypospermatocytogenesis or a significant increase in the number of spermatidic giant cells or spermatic granulomas was denoted as having testicular damage.

Experiment 2—Dose Response at Different Stages of Reproductive Development

At 29 d of age the animals were weight-ranked and placed into one of 3 groups according to their weight so that the mean body weight and standard deviation (SD) for each group were approximately the same. Animals in group 1 received benomyl treatments beginning at 33 d of age (prepubertal), group 2 at 54 d of age (pubertal), and group 3 at 75 d of age (postpubertal). Prior to the initial benomyl treatment, animals within each age group were again weight-ranked and placed into one of 5 benomyl-treatment groups, such that homogeneity of the mean body weights and SD existed between the treatment groups. A total of 26 animals were used in each age group, with 6 animals in the control group and 5 animals assigned to each benomyl treatment group.

Suspensions of benomyl in corn oil were prepared daily. Animals in each age group received 0, 125, 250, 500, or 1000 mg/kg·d by oral gavage (Table 1) for 5 d. Each animal was weighed prior to treatment, and daily

dosage was adjusted for body weight. Each day, half of the dose was administered between 8 and 9 a.m. and the remainder was administered between 1 and 2 p.m. Animals received the dosing solution each day at a rate of 1.0 ml/kg body weight.

At 29 d after receiving the last treatment, blood samples for hematological evaluation were collected from the tail vein. Red blood cell count (RBC), hematocrit (HCT), hemoglobin (HGB), mean cell volume (MCV), and white blood cell count (WBC) were determined using an electronic cell counter (Model ZBI, Coulter Electronics, Inc., Hialeah, Fla.). The accuracy of the counting system was tested with 4-C normal hematology Reference Control (Coulter Electronics, Inc., Hialeah, Fla.).

At 31 d after receiving the last treatment, animals in each age group were weighed and then killed using CO_2 gas. Cardiac blood and selected tissues identical to those in experiment 1 were collected. Sections of the epididymides and samples of luminal fluid to be used for sperm counting along with testicular samples for histological evaluation were prepared as described in experiment 1. The single postexposure observation time used in this experiment allowed evaluation of spermatozoa that were undergoing the early phases of spermiogenesis during exposure. Since FSH may serve as an indication of the spermatogenic function of the testes, sFSH analysis were conducted using a radioimmunoassay procedure (Niswender et al., 1969).

Statistical Analyses

All data were analyzed by a computer-based statistical package (SAS) containing the general linear models (GLM) regression and multivariate analysis of variance (MANOVA) procedures (Helwig and Council, 1979). Bartlett's test (Neter and Wasserman, 1974) was used to test for homogeneity of variances. The level of significance was set at $p < 0.05$ for all tests.

RESULTS

Experiment 1

Weights of selected tissues are reported in Table 2. No significant treatment differences were found in the weights of the liver, kidney, testes, seminal vesicles, or sections of the epididymides at any of the observation times. In addition, neither epididymal sperm counts (cauda or caput) nor vas deferens sperm concentrations (Table 3) were significantly related to the benomyl treatment. It may also be noted that the timing for the onset of puberty was not affected by benomyl treatment, as evidenced by the appearance of spermatozoa in the vas deferens by 61 d of age in both treatment groups.

TABLE 2. Selected Weights in Male Sprague-Dawley Rats after Prepubertal Exposure to Benomyl (Experiment 1)

Variable	Benomyl (mg/kg·d)	Age at necropsy (d) / Postexposure time (d)				
		47 / 3	61 / 17	75 / 31	89 / 45	103 / 59
Liver (g)[a,b,c]	0	11.6 ± 1.1	15.8 ± 1.7	16.8 ± 2.8	17.2 ± 2.0	18.4 ± 2.0
	200	12.2 ± 1.4	14.2 ± 1.7	15.7 ± 3.0	16.1 ± 1.3	17.3 ± 2.3
Kidney (g)[a,b,c]	0	2.25 ± .20	2.96 ± .26	3.06 ± .25	3.05 ± .25	3.39 ± .46
	200	2.26 ± .31	2.70 ± .24	2.98 ± .28	3.11 ± .32	3.44 ± .24
Testes (g)[a,b,c]	0	2.23 ± .31	2.92 ± .23	2.86 ± .26	3.20 ± .24	3.18 ± .23
	200	2.23 ± .23	2.84 ± .24	2.86 ± .26	3.08 ± .23	3.23 ± .25
Seminal vesicles (g)[a,b,c]	0	.296 ± .08	.832 ± .14	1.16 ± .28	1.18 ± .25	1.45 ± .27
	200	.356 ± .13	.761 ± .11	1.03 ± .11	1.27 ± .19	1.40 ± .17
Epididymis–caput (g)[a,b,c]	0	.178 ± .02	.377 ± .04	.478 ± .04	.541 ± .04	.592 ± .05
	200	.191 ± .02	.358 ± .03	.467 ± .06	.536 ± .03	.579 ± .02
Epididymis–cauda (g)[a,b,c]	0	.091 ± .01	.209 ± .02	.328 ± .04	.389 ± .03	.421 ± .04
	200	.090 ± .01	.186 ± .03	.290 ± .03	.386 ± .02	.415 ± .03

[a] Data presented as mean ± SD.
[b] Number of samples in each mean = 8.
[c] Significant age effect ($p < .05$) by GLM.

TABLE 3. Epididymal Sperm Counts and Vas Deferens Sperm Concentration in Sprague-Dawley Rats after Prepubertal Exposure to Benomyl (Experiment 1)

Variable	Benomyl (mg/kg·d)	Age at necropsy (d) / Postexposure time (d)				
		47 / 3	61 / 17	75 / 31	89 / 45	103 / 59
Epididymal (caput) sperm count, X 10^6 /pair[a,b,c]	0	4.6 ± 2.7	50 ± 12	71 ± 25	115 ± 32	112 ± 15
	200	4.0 ± 3.4	45 ± 8	66 ± 14	114 ± 19	111 ± 62
Epididymal (cauda) sperm count, X 10^6 /pair[a,b,c]	0	.64 ± 1.5	52 ± 27	118 ± 30	182 ± 48	170 ± 44
	200	.18 ± 2.8	47 ± 26	97 ± 27	201 ± 53	166 ± 47
Vas deferens sperm, X 10^6 /mg[a,b,c]	0	0	1.10 ± .27	1.46 ± .30	1.50 ± .19	1.71 ± .27
	200	0	1.09 ± .19	1.50 ± .29	1.53 ± .22	1.70 ± .33

[a] Data presented as mean ± SD.
[b] Number of samples included in each mean = 8.
[c] Significant age effect ($p < .05$) by GLM.

Experiment 2

Individual body weights of all animals observed daily during the exposure period indicated that the animals that received benomyl continued to gain weight at the same rate as the controls. In addition, no significant changes were found in the total body weight gain from the initial treatment to necropsy (36 d). Evaluation of hematologic parameters in these animals 29 d postexposure indicated that the RBC count, MCV, HCT, HGB, WBC count, or the calculated indices of mean corpuscular hemoglobins (MCH) and mean corpuscular hemoglobin concentration (MCHC) were unaffected by benomyl treatment in all age groups.

Weights of tissues associated with reproduction are reported in Table 4. When the data from all three age groups were analyzed together, a significant treatment effect was found in the cauda epididymides weights. A significant age-by-treatment interaction was noted in the testes and epididymides (cauda and caput) weights which indicated that the response to benomyl was not the same for each age group. When the data were analyzed by age, the testes and cauda epididymides weights were significantly depressed in animals that received benomyl during the onset of puberty.

Table 5 presents values for the epididymal (cauda and caput) sperm counts and vas deferens sperm concentrations. When the data were analyzed by age, the cauda epididymal sperm counts were depressed in the pubertal animals. While no significant linear or curvilinear treatment effects were found when using GLM to analyze caput epididymal sperm count or vas deferens sperm concentration at this age, heterogeneity of variance[1] was noted using Bartlett's test. This may reflect a tendency for an increased sensitivity of some animals in the age group to benomyl exposure. Animals that had received benomyl as adults had significantly depressed caput epididymal sperm counts. While the mean epididymal (cauda) sperm count and vas deferens sperm concentration for adult animals receiving the highest dose of benomyl (1000 mg/kg X 5 d) were depressed in comparison with the control means, no significant linear or curvilinear treatment effects were found using GLM. Heterogeneity of variance was found in these parameters and may again reflect increased sensitivity of some animals to benomyl exposure.

The sFSH levels (Table 4) were not significantly affected by benomyl treatment in the prepubertal or pubertal animals. However, individual animals in the pubertal group with low epididymal sperm counts and low vas deferens sperm concentration tended to have higher sFSH levels. sFSH levels in the adult animals were significantly increased with benomyl treatment.

[1] Homogeneity of variance is normally a requirement to insure the validity of the GLM procedure and *F*-test. However, due to the robust nature of this procedure, when unequal variances are present the *F*-test will be only slightly affected if the sample sizes for the dose groups are equal (Neter and Wasserman, 1974).

TABLE 4. Selected Tissue Weights and Serum FSH Levels in Prepubertal, Pubertal, and Postpubertal Male Sprague-Dawley Rats 31 Days after Receiving Five Oral Doses of Benomyl (Experiment 2)

Variable	Reproductive development during treatment	Benomyl (mg/kg·d)				
		0 ($N = 6$)[a]	125 ($N = 5$)	250 ($N = 5$)	500 ($N = 5$)	1000 ($N = 5$)
Testes[b,c,e] (g)	Prepubertal	3.04 ± .12	3.05 ± .20	2.89 ± .32	3.07 ±.27	3.29 ± .43
	Pubertal[f]	3.17 ± .23	3.22 ± .14	3.45 ± .26	2.94 ±.77	2.79 ± .46
	Postpubertal	3.47 ± .24	3.31 ± .41	2.65 ± .79	3.44 ±.54	3.24 ± .44
Seminal vesicles[b,c] (g)	Prepubertal	1.01 ± .24	.97 ± .06	.93 ± .09	1.03 ±.08	1.04 ± .10
	Pubertal	1.38 ± .26	1.42 ± .14	1.39 ± .21	1.43 ±.33	1.39 ± .19
	Postpubertal	1.73 ± .23	1.68 ± .33	1.42 ± .19	1.58 ± .28	1.48 ± .27
Epididymis—caput[b,c,e] (g)	Prepubertal	0.44 ± .02	0.46 ± .05	0.39 ± .06	0.46 ± .02	0.46 ± .03
	Pubertal	0.55 ± .04	0.58 ± .03	0.59 ± .07	0.49 ± .15	0.50 ± .12
	Postpubertal	0.65 ± .06	0.61 ± .07	0.47 ± .12	0.65 ± .05	0.61 ± .10
Epididymis—cauda[b,c,d,e] (g)	Prepubertal	0.25 ± .01	0.26 ± .02	0.25 ± .03	0.29 ± .03	0.26 ± .02
	Pubertal[f]	0.42 ± .03	0.42 ± .04	0.43 ± .07	0.36 ± .11	0.34 ± .08
	Postpubertal	0.49 ± .05	0.46 ± .05	0.34 ± .10	0.45 ± .04	0.37 ± .10
Serum FSH[b] (ng/ml)	Prepubertal	587 ± 168	614 ± 105	544 ± 128	603 ± 126	686 ± 41
	Pubertal	529 ± 117	565 ± 59	665 ± 135	623 ± 76	502 ± 81
	Postpubertal[f]	481 ± 86	399 ± 142	548 ± 139	564 ± 149	652 ± 162

[a] Number of samples.
[b] Data presented as mean ± SD.
[c] Significant age effect ($p < 0.05$) by GLM.
[d] Significant treatment effect ($p < 0.05$) by GLM when overall regression model (age, treatment, and age-by-treatment interaction) used.
[e] Significant age-by-treatment interaction ($p < 0.05$) by GLM when overall model used.
[f] Significant treatment effect ($p < 0.05$) by GLM when analyzed by age.

TABLE 5. Epididymal Sperm Counts and Vas Deferens Sperm Concentration in Prepubertal, Pubertal, and Postpubertal Sprague-Dawley Rats 31 Days after Receiving Five Oral Doses of Benomyl (Experiment 2)

Variable	Reproductive development during treatment	Benomyl (mg/kg·d)				
		0	125	250	500	1000
Epididymal (caput)[a,c,d] sperm count, X 10^6 / pair	Prepubertal	60 ± 12 (6)[b]	52 ± 22 (5)	49 ± 18 (4)	57 ± 25 (5)	67 ± 9 (5)
	Pubertal[f]	118 ± 3 (5)	112 ± 7 (5)	154 ± 66 (5)	72 ± 47 (5)	84 ± 45 (5)
	Postpubertal[e]	114 ± 18 (6)	122 ± 35 (5)	79 ± 30 (4)	117 ± 18 (5)	66 ± 57 (5)
Epididymal (cauda)[a,c] sperm count, X 10^6 / pair	Prepubertal	101 ± 20 (6)	92 ± 34 (5)	99 ± 38 (4)	113 ± 22 (5)	105 ± 9 (5)
	Pubertal[e]	237 ± 64 (5)	226 ± 62 (5)	211 ± 60 (5)	148 ± 116(5)	131 ± 69 (5)
	Postpubertal[f]	263 ± 48 (6)	253 ± 39 (5)	153 ± 141(2)	250 ± 18 (5)	191 ± 155(5)
Vas deferens[a] sperm concentration (X 10^6)/mg	Prepubertal	1.8 ± .20(6)	1.6 ± .43(5)	1.8 ± .40 (4)	1.6 ± .45 (5)	1.9 ± .21 (5)
	Pubertal[f]	1.7 ± .50(6)	1.6 ± .47(5)	1.7 ± .25 (5)	1.3 ± .67 (5)	1.6 ± .77 (5)
	Postpubertal[f]	1.9 ± .38(5)	1.8 ± .22(5)	1.4 ± .55 (4)	1.8 ± .18 (5)	1.4 ± .81 (5)

[a] Data presented as mean ± SD.
[b] Number of samples.
[c] Significant age effect ($p < 0.05$) by GLM.
[d] Significant age by treatment interaction ($p < 0.05$) by GLM when overall model used.
[e] Significant treatment effect ($p < 0.05$) by GLM when analyzed by age.
[f] Heterogeneity of variance indicated using Bartlett's test ($p < 0.05$).

An incidence report of testicular histological damage is shown in Table 6. The occurrence of focal hypospermatocytogenesis (1 to 5 affected tubules per cross section) is an incidental finding in rats (Bloom and Fawcett, 1975). However, diffuse hypospermatocytogenesis, and the occurrences of spermatidic giant cells and granulomas are indications of a more significant testicular lesion. Seminiferous tubules in the testes of animals with diffuse hypospermatocytogenesis were linked by a single layer of cells, germinal epithelium (spermatogonia), and Sertoli cells (Fig. 2 and 3). It is apparent that in these tissues primary spermatocytes were not being formed in the affected tabules at the time of necropsy. Diffuse hypospermatocytogenesis was noted in some animals from all three age groups. The number of occurrences and the severity tended to increase in those animals which received benomyl during the onset of puberty or during postpuberty. Three out of 4 affected animals in the pubertal group and 4 out of 8 affected animals in the postpubertal group were graded as having a moderately severe to high degree of diffuse hypospermatocytogenesis (i.e., only a few tubules per cross section had normal spermatogenesis occurring at the time of necropsy), as compared with 1 out of 2 affected animals in the prepubertal group. No corresponding testicular damage was observed in any of the animals in the control groups.

DISCUSSION

Data from experiment 2 are in agreement with experiment 1 in that no significant treatment effects were found in animals which received benomyl during the prepubertal stage of reproductive development. Animals that received benomyl just prior to the onset of puberty or during postpuberty tended to be more susceptible to the exposure. There appeared to be a fairly wide range of individual susceptibility in these two groups. Using Bartlett's test it was determined that heterogeneity of variance occurred in the epididymal sperm counts and in the vas deferens sperm concentrations. An example of this variation in values may be seen in the vas deferens sperm concentrations for animals that received the highest benomyl treatment (1000 mg/kg X 5 d) during postpuberty. Individual values ranged from 2.5×10^6/mg luminal fluid to 0.27×10^6, whereas the values for the controls in the same treatment group ranged from 1.66×10^6 to 2.44×10^6. Although no significant treatment effect was noted for the vas deferens sperm concentrations from the postpubertal animal, it is evident throughout the data that some of the animals were sensitive to the benomyl exposure. In such cases individual animals with low sperm counts also had high serum FSH values and some degree of testicular damage.

Histological examination of testicular tissue indicated a higher incidence of diffuse hypospermatocytogenesis in pubertal (20% of the treated animals) and postpubertal (40% of the treated animals) animals exposed to benomyl. These values were compared with those of prepubertal animals,

TABLE 6. Incidence of Histopathological Damage to the Testes in Male Sprague-Dawley Rats 31 Days after Receiving Five Oral Doses of Benomyl (Experiment 2)

Benomyl (mg/kg·d)	Number of animals observed	Reproductive development during treatment	Focal hypospermatocytogenesis[a]	Diffuse hypospermatocytogenesis[b]	Spermatidic giant cells[c]	Granuloma[d]
0	6	Prepubertal	0	0	0	0
125	5		0	1	0	0
250	5		0	1	1	1
500	5		1	0	0	0
1000	5		1	0	0	0
0	6	Pubertal	2	0	0	0
125	5		0	1	0	0
250	5		2	0	0	0
500	5		0	2	2	0
1000	5		1	1	0	1
0	6	Postpubertal	3	0	0	0
125	5		1	0	0	0
250	5		0	4	0	0
500	5		0	2	0	0
1000	5		2	2	0	1

[a]Occurrence of occasional seminiferous tubules lined by Sertoli cells only.
[b]Significant testicular damage with more than half of all seminiferous tubules affected.
[c]Presence of enlarged multinucleated spermatids.
[d]Presence of small lesions that consisted of localized accumulation of histocytes, lymphocytes, and plasma cells.

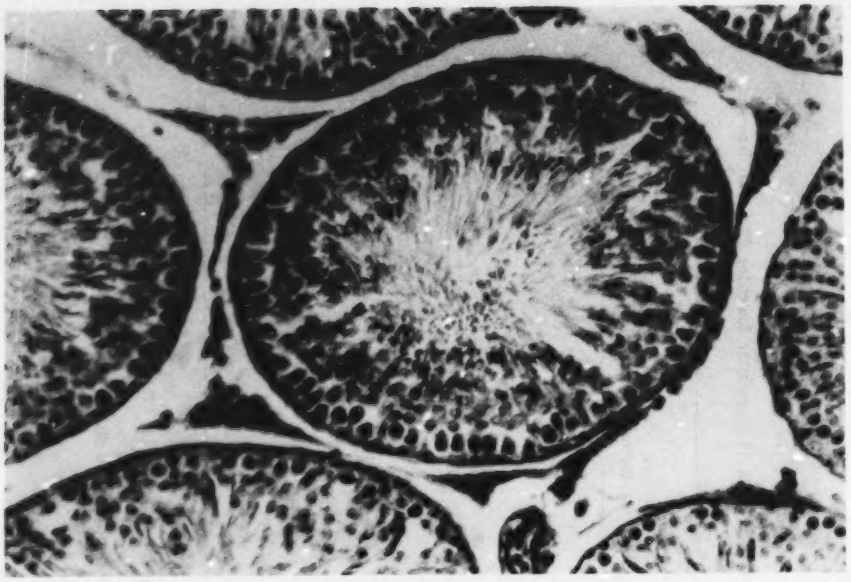


FIGURE 2. Cross section of postpubertal (110 d of age) control rat testis (0 mg benomyl/kg, X 5 d) with normal spermatogenesis occurring in all seminiferous tubules. PAS stain, X175.

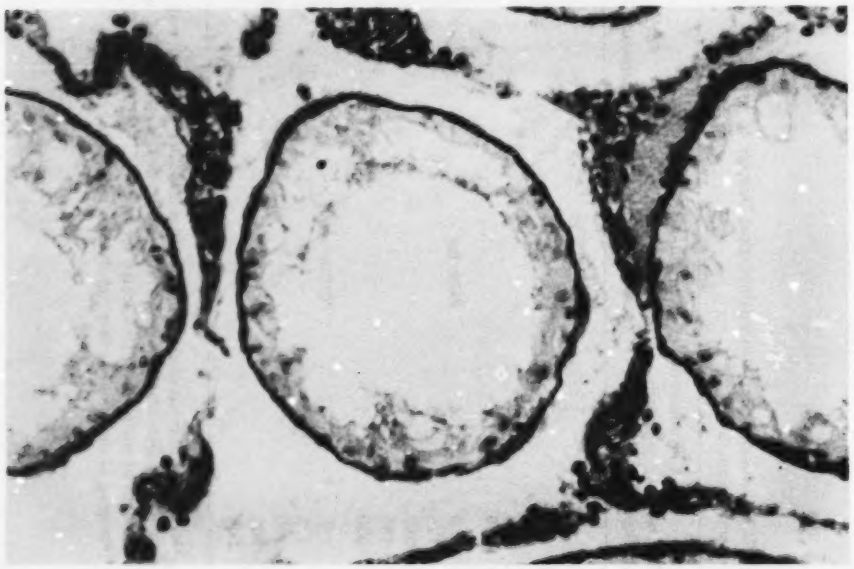


FIGURE 3. Cross section of postpubertal (110 d of age) treated rat testis after receiving 250 mg benomyl/kg·d from 75 to 79 d of age. Note diffuse hypospermatocytogenesis with only Sertoli cells remaining near the outer parameters of the seminiferous tubules. PAS stain, X175.

which had a 10% incidence of diffuse hypospermatocytogenesis, and with all of the control animals, which had no occurrences of testicular lesions.

Data from these experiments suggest that benomyl exposure during the pubertal and postpubertal stages of reproductive development in the male Sprague-Dawley rat may result in one or more of the following effects: decreased testicular or epididymal weights, decreased epididymal sperm count, decreased vas deferens sperm concentrations, and/or testicular lesions. This data is in agreement with results reported earlier by Carter and Laskey (1982), who noted a 32–48% reduction in vas deferens sperm concentration along with damage to the testicular germinal epithelium in adult Sprague-Dawley rats after 10 daily exposures to benomyl (200 or 400 mg/kg•d). The reproductive effects were more dramatic in the earlier report (Carter and Laskey, 1982), which may be a reflection of the longer exposure time. However, data from experiment 2, reported here, suggest that damage to the male reproductive system may occur during a shorter exposure (5 d) when exposure levels are at least 250 mg/kg•d.

Data reported here indicate that there is an age-related difference in sensitivity to benomyl. Animals exposed to benomyl during prepuberty appear to be less sensitive to the exposure than those that received the exposure during puberty or postpubertal. The precise reason for this difference in sensitivity is unknown.

REFERENCES

Bloom, W., and Fawcett, D. W. 1975. Male reproductive system. In *A Textbood of Histology*, ed. W. Bloom and D. W. Fawcett, pp. 805–857. Philadelphia: Saunders.

Carter, S. D., and Laskey, J. W. 1982. Effect of benomyl on reproduction in the male rat. *Toxicol. Lett.* 11:87–94.

Clermont, Y., and Perey, B. 1957. Quantitative study of cell population of seminiferous tubules in immature rats. *Am. J. Anat.* 100:241–267.

Davidse, L. C., and Flach, W. 1977. Differential binding of methyl benzamidazole-2-yl carbamate to fungal tubulin as a mechanism of resistance to this antimitotic agent in mutant strains of *Aspergillus nidulans*. *J. Cell. Biol.* 72:174–193.

Fox, R. R., and Laird, C. W. 1970. Sexual cycles. In *Reproductive and Breeding Techniques for Laboratory Animals*, ed. E. S. E. Hafez, pp. 107–122. Philadelphia: Lea and Febiger.

Friedman, P. A., and Platzer, E. G. 1978. Interaction of anthelmintic benzimidazoles and benzimidazole derivatives with bovine brain tubulin. *Biochim. Biophys. Acta* 544:605–614.

Gomes, W. R. 1970. Spermatogenesis. In *The Testis*, eds. A. D. Johnson, W. R. Gomes, and N. L. Vandemark, vol. I, pp. 339–411. New York: Academic.

Gomes, W. R. 1975. Endocrinology of the testis. In *The Testis*, eds. A. D. Johnson, W. R. Gomes, and N. L. Vandemark, vol. II, pp. 2–57. New York: Academic.

Gordon, D. L., Moore, D. J., Tnorshund, T., and Paulsen, C. A. 1965. The determination of size and concentration of human sperm with an electronic particle counter. *J. Lab. Clin. Med.* 65:506–512.

Hamner, C. E. 1970. The semen. In *Reproductive and Breeding Techniques for Laboratory Animals*, ed. E. S. E. Hafez, pp. 56–73. Philadelphia: Lea and Febiger.

Helwig, J. T., and Council, C. A. (eds.) 1979. *The SAS User's Guide*. Cary, North Carolina: SAS Institute Inc.

Humason, G. L. 1962. Fixation. In *Animals Tissues Techniques*, eds. G. W. Beadle, R. Emerson, and D. M. Whitaker, pp. 3–24. San Francisco: W. H. Freeman.

Ireland, C. M., Gull, K., Gutteridge, W. E., and Pogson, C. I. 1979. The interaction of benzimidazole carbamates with mammalian microtubule protein. *Biochem. Pharmacol.* 28:2680–2682.

Kavlock, R. J., Chernoff, N., Gray, L. E. Jr., and Whitehouse, D. 1982. Teratogenic effects of benomyl on the Wistar rat and CD-1 mouse with emphasis on the route of administration. *Toxicol. Appl. Pharmacol.* 62:44–54.

Laws, S. C., Rehnberg, G. L., Hein, J. R., and Laskey, J. W. 1983. Enumeration of rodent spermatozoa using an electronic cell counter. Abstract. *J. Androl.* 4(1):39.

Neter, J., and Wasserman, W. 1974. Implementation of ANOVA model. In *Applied Linear Statistical Models*, pp. 492–517. Homewood, Ill.: Richard D. Irwin.

Niswender, G. D., Midley, A. R., Jr., Monroe, S. E., and Reichert, L. E., Jr. 1968. Radioimmunoassay of Rat Luteinizing Hormone with Antiovine LH Serum and Ovine LH-131-I. *Proc. Soc. Exp. Biol. Med.* 128:807–811.

Olmstead, J. B., and Borisy, G. G. 1973. Microtubules. *Ann. Rev. Biochem.* 42:507–540.

Sherman, H., Culik, R., and Jackson, R. A. 1975. Reproduction, teratogenic, and mutagenic studies with benomyl. *Toxicol. Appl. Pharmacol.* 33(2):305–315.

Torchinskiy, A. M., Orlova, N. V., Smirnova, Y. B., and Makavova, L. F. 1976. Data on the toxicological and public health characteristics of the carbamate-group pesticide benlate. *Gig. Sanit.* 2:35–41.

Received June 1, 1983
Accepted August 24, 1983